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Daniel DENSHAM
36 Gabriel's Wharf
Water Lane
Exeter
EX2 4RN, United Kingdom

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — GB — 8673501001

4. Title of the invention — Method

5. Name of your agent *(if you have one)*

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

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11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature John Jappy Date 15 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

JAPPY, John William Graham
020 7377 1377

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# Method

## Field of the invention

Highly multiplexed monitoring of polynucleotide amplification reactions.

## Background of the Invention

A significant improvement in polynucleotide amplification, the polymerase chain reaction (PCR) technique, is disclosed in U.S. Pat. Nos. 4,683,202; 4,683,195; 4,800,159; and 4,965,188. In its simplest form, PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to the opposite strands and flank the region of interest in the target DNA. A repetitive series of reaction steps involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5'ends of the primers.

Recently, significant progress has been made in overcoming some of the problems of clinical diagnostic nucleic acid amplification through the development of automatable assays for amplified product that do not require that the reaction vessel be opened.

At the start of a PCR reaction, reagents are in excess, template and product are at low enough concentrations that product renaturation does not compete with primer binding, and amplification proceeds at a constant, exponential rate. Exactly when the reaction rate ceases to be exponential and enters a linear phase of amplification is extremely variable, even among replicate samples, but it appears to be primarily due to product renaturation competing with primer binding (since adding more reagents or enzyme has little effect). At

some later cycle the amplification rate drops to near zero (plateaus), and little more product is made.

For the sake of accuracy and precision, it is necessary to collect quantitative data at a point in which every sample is in the exponential phase of amplification (since it is only in this phase that amplification is extremely reproducible). Analysis of reactions during exponential phase at a given cycle number should theoretically provide several orders of magnitude dynamic range. Real-time PCR automates this otherwise laborious process by quantitating reaction products for each sample in every cycle. The result is a very broad 107-fold dynamic range, with no user intervention or replicates required.

All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. In one of the simplest formats, that reporter is a double-strand DNA-specific dye, known as SYBR ® Green (Patent No. ). SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases.

The advantages of SYBR Green are that it is relatively inexpensive, easy to use and sensitive. The major disadvantage to this system, however, is that SYBR Green will bind any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration.

Other commonly used alternatives to SYBR Green are TaqMan® and molecular beacons, both of which are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantitation.

TaqMan® probes are oligonucleotides that contain a fluorescent dye, typically on the 5'base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the S'exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

Molecular Beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement.

Multiplex reactions.

TaqMan® probes and molecular beacons allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogenous assays.

It can therefore be seen, that their exists a great number of inherent advantages within the process known as real-time PCR. Real-time PCR has increased sensitivity over traditional (endpoint) PCR. Traditional PCR has a relatively low (< 2 logs) dynamic range while real-time PCR as an extraordinary broad dynamic range (107 fold). Real-time or closed loop PCR does not require post PCR manipulations and reduces the possibility of cross contamination and increases the its ease in respect to automation. Real-time PCR allows detection of products down to a 2-fold change.

With these advantages, therefore, real-time PCR has been applied to a broad range of life science applications including: viral quantitation, quantitation of gene expression, array verification, drug therapy efficacy, DNA damage measurement, quality control and assay validation, pathogen detection and genotyping.

With all these inherent advantages, however, their still remains a number of inherent difficulties relating to the current state of the art real-time PCR that the current invention addresses. These drawbacks relate to the fact that almost all techniques applied to the process of real-time PCR relate to the use of fluorescent labels. As previously outlined herein, a number of fluorescent labels have been developed for use in the real-time quantification of PCR product. These fluorescent dyes must fur fill a number of stringent criteria. These dyes must be highly stable in chemical terms, both in terms of the amount of excitation light they can absorb and their ability to withstand the raised temperatures of the PCR process. The use of fluorescent dyes and fluorescent dye systems (e.g. FRET), further, puts important limitations on the sequence of DNA that may be used as a probe. This is due mainly to that fact that guanine residues in DNA are know to act as a quencher in a fluorescent resonant energy transfer process.

There, special care must be taken when selecting the sequences immediately adjacent to the emission dye, especially in systems directly utilizing the FRET process.

A further limitation of currently used fluorescent detection systems when applied in the contact of real-time PCR is the severe limitation that is placed on such a systems performance in terms of multiplexing and hence resultant sample throughput, especially in terms of high throughput screening (HTS) systems.

Multiplexing (using multiple primers to allow amplification of multiple templates within a single reaction) is a useful application of conventional PCR (Chamerliain, J.S. et al (1988) Nucleic Acid Res. 16:111141-11156) However, its transfer to real-time PCR has confused its traditional terminology. The term multiplex real-time PCR is more commonly used to describe the use of multiple fluorogenic oligoprobes for the discrimination of multiple amplicons. The transfer of this technique has proven problematic because of the limited number of fluorophores available (Lee, L. G et al (1993) Nucleic Acids Res. 21:3761-3766) and the common use of a monochromatic energizing light source. Although excitation by a single wavelength produces bright emissions from a suitably selected fluorophore, this restricts the number of fluorophores that can be included. Recent improvements to the design of the hairpin primers (molecular beacons), and hairpin and nuclease oligoprobes as well as novel combinations of fluorophores have been applied in an attempt to address this shortfall.

Early real-time PCR systems contained optimized filters to minimize overlap of the emission spectra from the fluorophores. Despite this, the number of fluorophores that could be combined and clearly distinguished was limited when compared with the discriminatory abilities of conventional multiplex PCR. More recent real-time PCR platforms have incorporated either multiple light-

emitting diodes to span the entire visible spectrum, or a tungsten light source, which emits light over a broader range of wavelengths. When these platforms incorporate high quality optical filters it is possible to apply any current real-time PCR detection chemistries on the one machine. Nonetheless, these improvements generally allow only four-colour oligoprobe multiplexing, of which one colour is ideally set aside for an internal control to monitor inhibition and perhaps even act as a co-amplified competitor.

There is therefore a need for an improved method for monitoring nucleic acid amplification in a highly multiplexed manner and which is preferably carried out by an automated process, reducing the complexity and cost associated with existing methods.

## Summary of the Invention

The present invention is based on the realization that the progress of a nucleic acid amplification reaction may be monitored by detecting the interaction between amplified polynucleotide and a spatially localized probe molecule or molecules.

According to one aspect of the present invention, a method for monitoring nucleic acid amplification comprises the steps of:

(i) performing nucleic acid amplification on a polynucleotide using a polynucleotide processive enzyme under conditions suitable for enzyme activity; and

(ii) detecting the interaction between amplified polynucleotide(s) and immobilized probe molecule(s).

In a preferred embodiment, the enzyme is a polymerase, typically a thermostable polymerase which takes part in a polymerase chain reaction (PCR).

In a further preferred embodiment the enzyme is a reverse transcriptase, typically a thermostable reverse transcriptase, which takes part in a reverse transcriptase polymerase chain reaction (RT-PCR).

According to a second embodiment of the invention, the probe molecule is localized in space via immobilization on a solid support.

In one preferred embodiment of the present invention, the detection of interaction between the amplified polynucleotide molecule and the localized probe molecule occurs via the use of surface electromagnetic waves (SEW).

In a preferred embodiment of the present invention the surface electromagnetic wave (SEW) is in the form of a plasmon, known as a surface plasmon. Thus the solid support utilized within the context of the present invention is one in which is known within the art to support the propagation of surface plasmons (e.g. Ag, Au).

According to a further aspect, a solid support comprises at least one immobilized probe molecule capable of interacting with an amplified polynucleotide sequence.

The use of surface electromagnetic waves within the context of the present invention offers several advantages over conventional real-time PCR technology. Surface electromagnetic wave technologies are generally utilized without the need for "labels". Current fluorescence based systems place restrictions on the sequence of the oligonucleotide probe that may be used to interrogate the amplified polynucleotide product. This is due to quenching and secondary structure effects upon and relating to the use of fluorescent label systems.

In another preferred embodiment of the present invention, oligonucleotides are used as the probe molecules.

In a further preferred embodiment oligonucleotides are immobilized on a solid support and interaction/hybridization with amplified polynucleotide is detected using fluorescence means (this can be with either intercalating dyes, immobilized fluorescent molecular beacons or immobilized TaqMar® probes).

Description of the Invention

The present method for monitoring the progress of a nucleic acid amplification reaction involves the analysis of the interaction between an amplified polynucleotide and spatially localised probe molecule or molecules.

The term "polynucleotide" and/or "oligonucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridizing nucleic acid-like molecules, e.g. peptide nucleic acid (PNA).

The "probe molecule" may be any molecule which interacts with a polynucleotide, either double or single stranded. In a preferred embodiment of the present invention the probe molecule is a molecule which interacts with a specific polynucleotide sequence to as to selectively probe for such a sequence during the progress of polynucleotide amplification. Such a sequence specific binding molecule may be any know type. Preferred molecules include polynucleotide binding proteins such as double and single stranded binding proteins. Such proteins may be recombinant proteins as known within the art which contain site specific polynucleotide binding domains. Such domains are well known within the art (Duncan et al, (1994) Genes Oev. 8(4):465-80).

In a preferred embodiment of the present invention, the probe molecule(s) consists of single stranded oligonucleotides. Such single stranded oligonucleotides therefore act as hybridization targets for amplified polynucleotide sequences, in an analogous way to the fluorescently labeled probe sequences used within conventional real-time PCR approaches.

In a further preferred embodiment of the present invention, "standard" fluorescent molecular beacons are immobilized on a solid support.

In yet another preferred embodiment of the present invention, interaction between the amplified polynucleotide and the molecular probe molecular is achieved via the use of an intercalating dye molecule(s).

In a preferred embodiment of the present invention, hybridization between a spatially localised probe molecule and an amplified polynucleotide is detected/monitored by the use of an intercalating fluorescent dye. Such dyes are well know in the art and include Syber

In a preferred embodiment, a number of oligonucleotide sequences are used to probe the amplification products. Such an embodiment may be envisioned as an array of oligonucleotides. In a preferred embodiment the probe molecules are immobilized on a solid support supporting a surface electromagnetic wave. It is further preferred that the surface electromagnetic wave be a surface plasmon wave. It is envisioned that any number of surface plasmon detection modalities may be used within the context of the present invention. Conventional total internal reflection geometries are envisioned within the scope of the current invention.

In a further preferred embodiment of the current invention, a preferred detection modality for the surface plasmon wave involves the use of

interfereometry. Such interferometric techniques are well known in the art and any known technique is viewed as within the scope of the current invention. Applicable interferometric techniques include, but are not limited to, systems outlined in GBO2/003543, WO 93/14392, WO 95/22754 and WO 01/20295.

In a preferred embodiment of the present invention, the interference pattern produced by changes on the SPR supporting surface (and hence a measure of interaction with the polynucleotide probe molecule and therefore a measure of the amplification reaction) is produced by the interaction of two source beams. One source beam originating from scattering from one end of a plasmon supporting nanostructure (reference beam) and the other beam produced by scattering from the far end of the structure, the second beam produced after traveling from the first beam origin as a surface electromagnetic wave. Such a set-up is outlined in the co-pending patent application GB 0220341.2.

In a preferred embodiment of the present invention, the set-up utilized in GB 0220341.2 is further modified as outlined wihin said natent such that the anale of the window allowino the exit of the two interfering beams from the liquid cell is adjusted so as to minimize the influence of bulk refractive index changes within the fluid/reactant medium. In this configuration, it is shown that for any particular interference minimum there is a window angle at which any variations of the RI of the liquid the position of the minimum on the detector will not be changed. This setup has the advantage, within the context of the present invention, of suppressing RI (signal) changes as a result of changes in liquid/reactant temperature. Thus, as the PCR process is carried out, the changes in SEW/SPR signal experienced as a result of thermo cycling will be

eliminated/reduced. This has obvious major benefits within the context of the present invention.

In a further embodiment of the invention, changes in signal from the SEW/SPR surface due to changes in tern perature/thermo cycling are mimimised/surpressed by measuring the temperature within the reaction chamber and correcting the output data using a software algorithm. In yet a further envisioned embodiment, window angle adjustment and online temperature measurement/software adjustment are combined to minimized signal fluctuation due to temperature changes/cycling.

In a preferred embodiment of the present invention, the probe molecules are immobilized at discrete sites across the solid support such that determination of the identity of a specifically binding/interacting polynucleotide sequence is via localization in space (as in array formats).

In yet a further preferred embodiment of the present invention, hybridization of an amplified polynucleotide sequence to immobilized oligonucleotide is monitored throughout the amplification process, i.e. during all phases of temperature cycling as carried out within conventional PCR. Thus the temperatures at which hybridization and melting of each probed sequenced maybe monitored throughout the reaction. Thus, in a preferred embodiment of the present invention, the concentration of the PGR reactants and products may be determined by monitoring a signal generated by the hybridization of an immobilized oligonucleotide and amplified polynucleotide sequences during temperature cycling. In a preferred embodiment, relative SEW data is collected at a point in which every sample is in the exponential phase of amplification (since it is only in this phase that amplification is extremely reproducible).

In a preferred embodiment, a real-time PCR system is created that relies on the detection and quantification of a SEW/SPR hybridization signal (either intensity and/or interferometric), the signal of which increases in direct proportion to the amount of PCR product in a reaction.

In the simplest and most economical foreseen PmhAdimpnt oligoruicleotide seouences are immobilized in an SEW/SPR array format and interactions between these oligonucleotides and PCR products are monitored throughout the phases of temperature cycling that constitute the PCR process.

In a preferred embodiment, interaction data obtained during the exponential phase of each thermo cycle is compared in order to quantify the amount of template in a reaction and/or kinetic data during a reaction and/or sequence identity/modification.

In a preferred embodiment, the PCR reaction is carried out within a sealed micro-flow cell. Once the reactants are introduced into the flow cell (which is created above the SEW sensor surface), the input/output valves are closed and a sealed flow cell system is created. In such a sealed flow cell system, and integrated pump keeps the reactionlPCR fluid flowing in a closed circuit. One part of the circuit flows the reactant mixture over the sensor surface with its immobilized probe molecules. During the PCR reaction, thermo cycling is carried out by changing the temperature of the entire flow cell/sensor surface assembly.

In a further embodiment of the present invention, primer and/or probe sequences for the PCR reaction are attached to plasmon supporting particles - such as gold spheres and allowed to move freely in solution. Complimentary sequences to these primer sequences are immobilized as already outlined on

a SEW supporting solid support. As the PGR reaction proceeds, more and more of the primer/probe sequences will bind to the increasing amount of product polynucleotide, leaving less primer/probe available to bind to their complements on the solid support. Monitoring the SEW/SPR signal during the PCR reaction will give an indication of the reaction constituents. It is preferred that the free primers/probes are labeled with a plasmon active label (such as a gold sphere) but it is also foreseen that these sequence do not need to be so "labeled".

In a further foreseen embodiment, the probe molecules immobilized are known as molecular beacons. A molecular beacon is based on a polynucleotide that takes a hairpin formation, and that if separated into single strands, has five or more complimentary bases at each end of a strand. A 30-base sequence, for example, might be GCTTA - (20 other bases here) - TAAGC. If this single strand can't find a complementary strand, the DNA will fold itself in half, into a hairpin shape. In conventional molecular beacons, one end of the strand has a fluorescent molecule attached to it and the other has a quenching agent. In a conventional reaction, therefore, if a complementary single strand is introduced to the beacon, the hairpin unfolds and attaches to it, separating the fluorophore and its quencher and allowing fluorescence and detection. In the preferred embodiment of the present invention, however, one end of the hairpin is attached to the SEW/S PR supporting film (i.e. gold) and the other end is attached to a plasmon supporting nanostructure (e.g. gold particle). When the hairpin is subjected to a complementary polynucleotide sequence, it will unfold moving the particle away from the surface. As the particle moves away from the surface, its interaction with the surface electromagnetic wave will alter (as such waves are strongly localized down to the nanometer level) and a signal change will be detected.

In a further foreseen embodiment of the present invention, the immobilized oligonucleotides are labelled with plasmon supporting nanostructures (e.g gold nanoparticles). These nanostructures serve to enhance signals generated as a result of hybridization.

In a further preferred embodiment, intercalating molecules are linked to particles able to support plasmonic and/or resonant modes and/or able to undergo surface enhanced raman scattering. Such particles include, but are not restricted to, gold or silver spheres, cylinders or geometrical shapes varying in size between 0.5nm to several microns. In this way the formation of duplex polynucleotide structures at the localized hybridization locations will result and a greatly enhanced signal.

Detection may also be carried out in solution phase in which raman scattering is used as a measure of polynucleotide- probe molecule interaction.

In a further preferred embodiment of the present invention an electromagnetic filed is combined with the detection protocol (e.g. SPR, SEW, fluorescent) in order to affect the kinetics of hybridization (ideally speed up) and allow greater elucidation of mismatch structures.

CLAIMS

1. A method for monitoring nucleic acid amplification comprising:-

(i) Performing nucleic acid amplification on a polynucleotide, using a polynucleotide processive enzyme tinder conditions suitable for enzyme activ y, in which a probe sequence is localtsed in space, and;

(ii) Detecting the interaction between the localised probe molecule and the amplification products.

2. The method of claim 1, wherein said interaction is monitored via the use of a surface electromagnetic wave (SEW).

3. Method according to claim 2, wherein said SEW is a surface plasmon wave and detection is carried out by surface plasmon resonance.

4. Method according to claim 3, in which the mode of registration of surface plasmon resonance used is phase registration,

5. A method according to claim 4, in which the change in refractive index of the surface due to fluctuations in temperature is minimized by allowing both a reference and a secondary beam to travel through the reaction mixture.

8. A method according to claim 5, wherein the change in refractive index due to temperature fluctuations is further suppressed by controlling the exit angle of the two beams as they leave the reaction chamber

7. A method according to claim 1, in which a proportion of the primer is free in solution while a further proportion of said primer is Immobilized on a solid support.

8. A method according to claim I in which the probe is an oligonucleotide.

9. A method according to claim 1, in which the probe is a molecule capable of selectively binding to a polynucleotide.

10. A method according to claim 9, in which the molecule is a protein.

11. A method according to claim 9, in which the molecule is a single-stranded binding protein.

12. A method according to claim 9, in which the molecule is a polynucleotide processive enzyme.

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